Photoreactivation of Transforming DNA by an Enzyme from Bakers' Yeast

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ABSTRACT Ultraviolet-inactivated *Hemophilus influenzae* transforming DNA recovers its activity when mixed with cell-free extracts of bakers' yeast and exposed to visible light. The active agent in the extract is not used up in the reaction, and purification has not separated it into more than one non-dialyzable component. It differs from the agent in *Escherichia coli* extract, which produces very similar photoreactivation, but which can be resolved into non-dialyzable and dialyzable components, the latter being used up during illumination.

The yeast agent can be salted out of solution and recovered quantitatively; it is inactivated by crystalline trypsin and chymotrypsin and by brief heating at 60°C.—all facts suggesting that it is an enzyme for which ultraviolet lesions in the DNA serve as substrate. The kinetics of recovery are also consistent with such an assumption.

This enzyme is unusual both because it is involved in a light-dependent reaction and because it has a non-destructive action on DNA outside an intact cell.

INTRODUCTION

The effects on many organisms of 250 to 300 mμ ultraviolet radiation are markedly diminished by subsequent illumination at longer wave lengths. This phenomenon, known as photoreactivation (PR), has been studied for nearly a decade in a variety of different cells and for a number of ultraviolet effects. The over-all evidence, as recently reviewed by Jagger (1), suggests that ultraviolet radiation damage to nucleic acid (or possibly to protein, or both) is repaired by the cell upon exposure to the longer wave length light.

It was shown in previous work (2, 5) that bacterial transforming DNA, which has been inactivated by ultraviolet light, can be photoreactivated *in vitro* provided it is mixed with an extract of *Escherichia coli* B before the illumi-

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nation. Several features of this process suggest that it is related to the reactivation observed in intact cells. The ultraviolet dose required to inactivate this DNA is of the same general order of magnitude as that required to kill microorganisms—a dose far smaller than that required to change the absorption spectrum or viscosity of DNA solutions (3, 4). The organism, *E. coli* B, from which the necessary cell-free extract is derived is one which itself photoreactivates under the proper conditions. Similar extracts of *Hemophilus influenzae*, an organism which does not photoreactivate, are ineffective in spite of the fact that it is *H. influenzae* transforming DNA which is employed in the experiment (5). The light dose required for recovery of the transforming activity, the maximum degree of recovery obtained, and the dependence of recovery rate on the temperature are all similar to those found in the photoreactivation of intact *E. coli* cells.

This *in vitro* photoreactivation system is much simpler for study than its *in vivo* analogue. Both the inactivation and the subsequent photoreactivation occur in solutions containing only cell-free components. The "survival" observed is that of individual genetic markers being incorporated into an organism during bacterial transformation rather than the survival of a whole cell. The inactivation, reactivation, and measurement of marker survival are separate operations which can be delayed relative to each other by any length of time desired. This relative simplicity and freedom of manipulation open new possibilities in the investigation of the subject.

As shown in the earlier work, the active portion of the *E. coli* extract does not sediment in 1 hour at 109,000 $\times$ g, indicating that it is probably in solution. The extract loses activity upon dialysis and recovers it upon the addition of concentrated dialysate, the non-dialyzable portion being heat-labile while the dialyzable portion is not. The maximum level of recovery decreases as the amount of concentrated dialysate in the reconstituted mixture is diminished, suggesting that this dialysate contains a component which is used up during the reaction.

Since these earlier studies, it has been found that extracts of bakers' yeast (another organism capable of photoreactivation) will also provide for photoreactivation of transforming DNA (6). The yeast system shares many properties with the one found in *E. coli*, but shows no evidence of a component which is consumed during photoreactivation. The crude extract has, moreover, a higher activity than *E. coli* extract; it gives less trouble from active nucleases; and the starting material is inexpensively available in quantity. Photoreactivation by yeast extract thus represents an additional step toward simplicity and freedom of manipulation.

The present paper will be concerned with the characteristics of this second photoreactivation system.

Materials and Methods

The preparation of *H. influenzae* transforming DNA for streptomycin resistance (SrDNA), its ultraviolet inactivation (to form UV SrDNA), and the methods for assaying its transforming activity are essentially those described previously (2), with the following minor modifications.

DNA stocks were diluted and irradiated at 1.5 γ/ml. in 0.15 M saline. The usual reaction mixture contained two volumes of DNA solution combined with one volume of diluted yeast extract to give 1 γ DNA/ml. When samples of this mixture were mixed with competent *H. influenzae* cells for transformation, a 30-fold dilution occurred, giving usually a concentration of 0.033 γ DNA/ml. in contact with the cells. In some experiments, when specifically noted, the samples of a reaction mixture were diluted before assay to give a lower concentration than this.

Wherever the transformation procedure is identical with that described previously (2), the data carry the notation "TFM by layer method." This procedure was altered in some of the experiments reported here to save manipulation time and to provide a more uniform drug dose to the transformed cells—a measure which is necessary in working with some of the less convenient genetic markers. In the modified procedure (due to Dr. S. H. Goodgal)[1] the competent cells were incubated in contact with the DNA in Levinthal broth for 2 hours, and then plated directly in medium containing 500 γ/ml. streptomycin, instead of being exposed to DNA for 30 minutes, plated in non-selective medium, and then layered after 2 hours more with agar containing the streptomycin. (Delayed application of the drug is necessary to permit the transformed cells to develop the new characteristic.) The altered method is equivalent to the other in all its essentials. When highly competent *H. influenzae* cultures are diluted into fresh medium, they lose the power to take up appreciable amounts of DNA after the first half-hour and do not regain it until after the logarithmic phase of growth (8, 9). Even then they do not ordinarily recover to their former high level, since this requires special growth conditions (9). Hence, the DNA uptake in the altered procedure occurs only during the first half-hour as before. Furthermore, the number of cells in the newly transformed culture which are capable of developing the new characteristic (and, therefore, of giving rise to drug-resistant colonies) does not change for about 90 minutes after DNA uptake (7, 9) (although the total number of cells in the culture increases throughout this period). The number of transformants observed after 2 hours is, therefore, simply the number which would be observed using the older method multiplied by a small factor. It is important that all tubes in an experiment be treated identically if this multiplication factor, which is approximately 2, is to be the same for all.

The raw result of a transformation assay of DNA is the number of transformants per milliliter (TFM/ml.) produced by the sample of DNA in the transformation tube. Such numbers are strictly comparable only within one experiment since they depend not only on the concentration and condition of the DNA sample but also on the

[1] This procedure was adapted from a test for transformation described by Alexander and Leidy (7).

number of cells treated with it and on the susceptibility of the latter to transformation (their "competence"). This last factor varies somewhat from one experiment to another in the work reported here. Usually about one out of two hundred cells was transformed when using an excess of SrDNA.

Photoreactivation mixtures were illuminated by a bank of three closely spaced, parallel, "cool white" fluorescent tubes placed approximately 3 cm. below a glass-bottomed temperature bath. Screw-capped test tubes containing the reaction mixtures rested upright on the glass bottom directly over the center fluorescent tube.

Yeast extract was prepared by the Lebedew technique (10). Fresh, compressed yeast was crumbled, thoroughly air-dried, and slowly stirred into three times its own weight of 0.066 M Na_2HPO_4 to form a smooth, creamy suspension. This mixture was incubated 4 to 5 hours at 37°C. and then cleared of cellular debris by centrifugation. In some cases a final clarification was effected by filtration using Celite analytical filter aid (Johns Manville Corporation).

Active extract could also be obtained by mechanically rupturing fresh yeast cells and centrifuging, but not by plasmolysis in toluene followed by extraction with water or 0.066 M Na_2HPO_4.

Unused extract was stored at −20°C.

EXPERIMENTAL

Photoreactivation by Yeast Extract

When a mixture of ultraviolet-irradiated transforming DNA and diluted yeast extract is illuminated and sampled at intervals, the transforming activity progressively increases as seen in Fig. 1. This rise is characterized by an initial lag and a final plateau level which represents less than 100 per cent of the original activity.

Recovery proceeds only during illumination and ceases for the duration of the dark period. Upon resumption of illumination the recovery resumes at approximately the same rate at which it left off with no recurrence of the initial lag, as shown in Fig. 2.

The increase in transforming activity produced by illumination is observed only when the transforming DNA has first been ultraviolet-irradiated. The extent of photorecovery of irradiated material is the same no matter what concentration of DNA is employed to test for transforming activity. These facts are illustrated by Fig. 3, where mixtures of irradiated or unirradiated DNA with yeast extract have been sampled before and after an illumination period, and the samples tested for transformation at a number of different dilutions. The points representing unirradiated, irradiated, and photoreactivated DNA on this log-log plot are all fitted by parallel curves, both when recovery proceeds from 1 per cent to 20 per cent of the original activity, as shown here, and when it is stopped at 2 to 3 per cent (a separate experi-

ment). It was previously found (2) that the per cent activity of irradiated transforming DNA is independent of the DNA concentration employed in assaying. Fig. 3 shows that the same is at least approximately true of photoreactivated DNA as well.

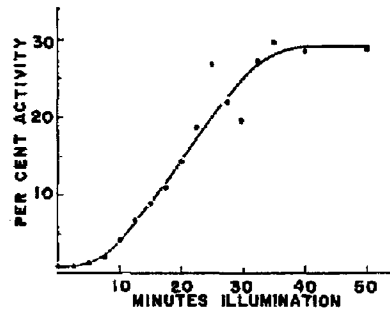

FIGURE 1. Photoreactivation of *H. influenzae* transforming DNA (streptomycin resistance marker) by dilute yeast extract. The number of transformants per milliliter produced by samples of the reaction mixture taken after various illumination times is expressed as per cent of the number produced by a control mixture containing unirradiated transforming DNA (TFM by layer method).

Photoreactivation occurs only when the irradiated DNA and yeast extract are mixed together at the time of illumination. As indicated in Table I, the addition of ultraviolet-irradiated DNA immediately after termination of an extended illumination period gives no increase as compared with addition of the same DNA to yeast extract preincubated in the dark. Subsequent illumination of either mixture, however, produces photoreactivation.

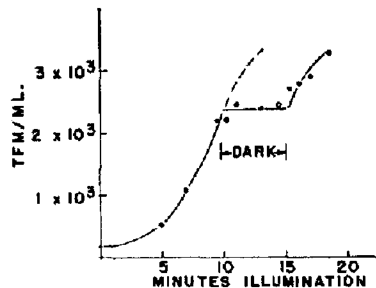

FIGURE 2. Photoreactivation with interrupted illumination (TFM by layer method).

The time rate of photoreactivation increases with temperature during illumination, as illustrated by recovery curves for two temperatures in Fig. 4. The extract is, however, inactivated by heating to 60°C. for 20 minutes, as shown in Table II.

Besides photoreactivation of the streptomycin resistance marker, analogous photorecovery has been observed with markers for cathomycin and viomycin resistance and for two different levels of erythromycin resistance.

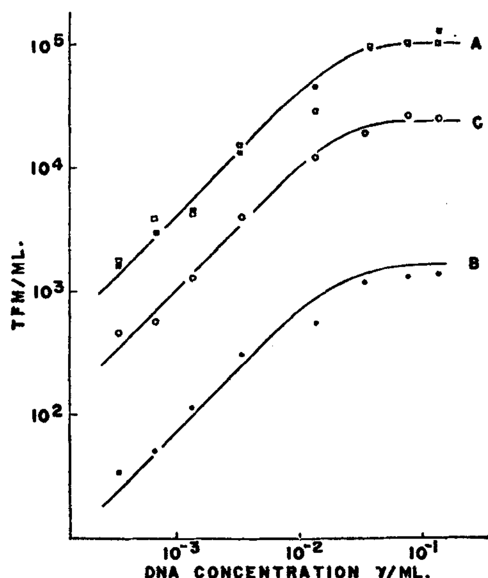

FIGURE 3. Titration curves of transforming DNA in mixtures with yeast extract before and after photoreactivation. Samples of these mixtures were diluted and tested for transforming activity. The abscissa is the DNA concentration in contact with *H. influenzae* cells in the transformation tube, while the ordinate is the number of transformants per milliliter corresponding to this concentration. Curve A, unirradiated DNA with yeast extract before illumination (solid squares) and after illumination (open squares). Curve B, irradiated DNA and yeast extract before illumination. Curve C, irradiated DNA and yeast extract after illumination (TFM by layer method).

TABLE I

FAILURE OF PREILLUMINATION TO PRODUCE REACTIVATION WITH YEAST EXTRACT

Tube	Contents and treatment at 37°C.	Transformants/ml. produced by sample
1	Yeast extract (a partly purified preparation) was incubated 20 min. under fluorescent illumination. U.V.-inactivated transforming DNA was added and mixed within 3 sec. of light extinction. Mixture was further incubated 5 min. dark. Sampled in triplicate	2.3×10^2 2.1×10^2 2.1×10^2
	Mixture was further incubated 20 min. under fluorescent illumination. Sampled	3.0×10^3
2	Partially purified yeast extract was incubated 20 min. dark. U.V.-inactivated transforming DNA was added. Mixture was further incubated 5 min. dark. Sampled in triplicate	2.1×10^2 2.3×10^2 2.1×10^2
	Mixture was further incubated 20 min. under fluorescent illumination. Sampled	3.2×10^3

TFM by layer method.

TABLE II

HEAT LABILITY OF YEAST PHOTOREACTIVATING AGENT

Crude yeast extract was diluted tenfold in 60°C. 0.005 M Tris buffer at pH 8 and held at this temperature for 20 minutes. For the unheated control, the same extract preparation was diluted in cold Tris buffer. Reaction mixtures were made using 2 volumes of UV SrDNA (1.5γ/ml.) and 1 volume of diluted extract preparation, sampled and incubated at 37°C. light or dark as indicated below.

Extract preparation in reaction mixture	Incubation conditions	Transformants/ml produced by samples after t min. incubation		
		$t = 0$	$t = 20$	$t = 40$
Heated extract	Light	3.8×10^2	4.3×10^2	3.8×10^2
Heated extract	Dark	3.6×10^2	—	4.5×10^2
Unheated extract	Light	3.9×10^2	8.4×10^3	6.0×10^3

In all the above features (except the initial lag, which will be considered later) photoreactivation by yeast extract resembles that produced by *E. coli* extract (2).

Effect of Extract Concentration

The rate of recovery diminishes with increasing dilution of the yeast extract in the reaction mixture, but the curves still tend to the same final plateau. Fig. 5 shows the course of recovery for three different concentrations of crude extract and two concentrations of an ammonium sulfate fraction of that

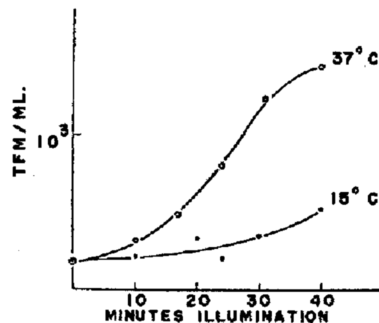

FIGURE 4. Effect of temperature during illumination on photoreactivation by yeast extract (TFM by layer method).

extract. These curves are all related in a simple way which can be seen by employing a logarithmic time axis, as in Fig. 6. Here the replotted curves of Fig. 5 become parallel segments of a single curve, each segment being simply displaced by a different amount along the logarithmic time axis. In Fig. 7 these segments have been made to lie along a single curve by translating each through the appropriate horizontal distance.

It is evident that the curves of Fig. 5 all belong to a single family in which

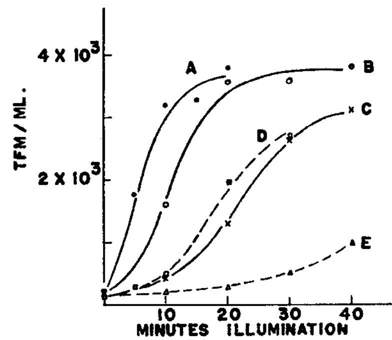

FIGURE 5. Effect of yeast extract concentration on photoreactivation of UV SrDNA. Solid curves are for experiments with crude yeast extract. Curve A, fourfold diluted. Curve B, tenfold diluted, Curve C, 20-fold diluted. Dashed curves are for an ammonium sulfate fraction (dialyzed to remove traces of ammonium sulfate). Curve D, undiluted. Curve E, fivefold diluted (TFM by layer method).

T, the number of transformants produced, is related to the illumination time, t, by $T = F(rt)$. The same function, F, applies to all, and only r, having the dimensions of rate, differs from curve to curve. This rate is approximately proportional to extract concentration in the concentration range employed here.

While the function, F, does not change with the amount or purity of the yeast photoreactivating agent, it does change with the radiation dose delivered to the DNA as will be seen below.

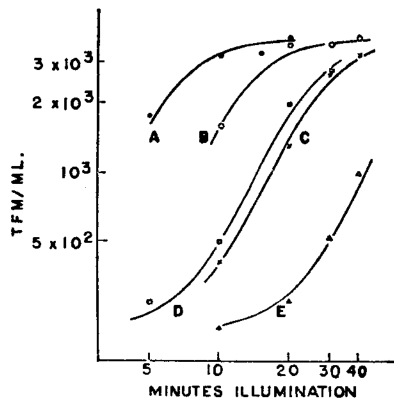

FIGURE 6. Data of Fig. 5 replotted on a logarithmic abscissa and ordinate.

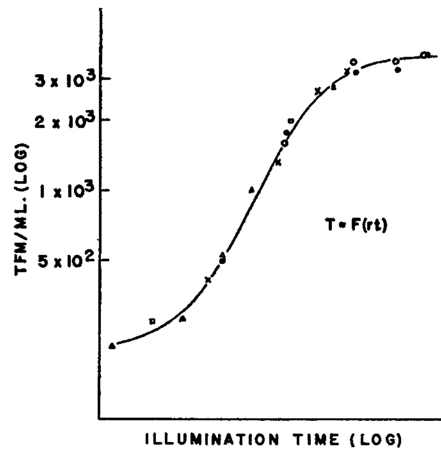

FIGURE 7. Curves of Fig. 6 superimposed by translation along the logarithmic time axis.

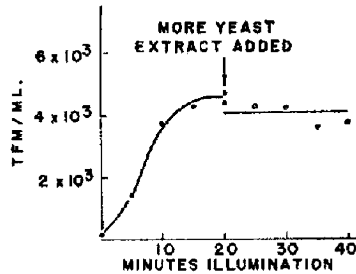

FIGURE 8. Effect of adding more yeast extract after photoreactivation to maximum (TFM by layer method).

Lack of Evidence for a Stoichiometric Component

The height of the recovery plateau is not the result of exhausting the photoreactivating power of the extract, as shown by Figs. 8–10. In Fig. 8 the addition of fresh yeast extract after photoreactivation to maximum produces no

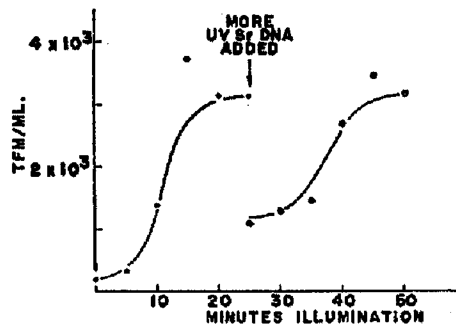

FIGURE 9. Photoreactivation of additional irradiated DNA after photoreactivation to maximum of an initial specimen. Samples assayed at a concentration of 0.033 γ DNA/ml. in contact with *H. influenzae* cells (corresponding to the plateau level of the titration curves of Fig. 3) (TFM by layer method).

further increase. However, in Figs. 9 and 10 the addition of more irradiated DNA at this time (in an amount approximately equal to that already in the mixture) results in a second round of photoreactivation. This second round is observed both when the samples of the reaction mixtures are assayed at "high" concentration (0.033 γ/ml. in the transformation tube, as in Fig. 9)

and at "low" concentration (1/30 of this level, as in Fig. 10). The different appearance of the recovery curves in these two cases is just that which would be expected if the added DNA is restored in the same manner as the original DNA.

As pointed out by Hotchkiss (11), the number of transformants at high DNA concentration "is a measure of the *quality* of a DNA preparation—its ability to supply active DNA bearing a certain marker relative to its total

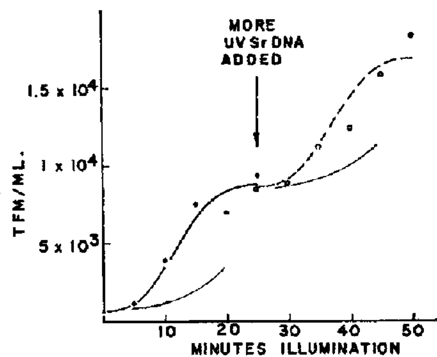

FIGURE 10. Assay of the same samples shown in Fig. 9 after 30-fold further dilution. The resulting DNA concentration corresponds to the linear portion of the titration curves in Fig. 3. The number of transformants per milliliter is higher than in Fig. 9 because, for convenience, more *H. influenzae* cells were used to test the transforming activity of these diluted samples (TFM by layer method).

DNA content." In Fig. 9, the reduction in the number of transformants (to about half) which is produced by adding inactive DNA reflects the fact that the fraction of active molecules in the solution is approximately halved by this addition. It is the result of competition between the active and inactive molecules for uptake by the cell. The fact that the second half of the curve rises to approximately the same level as the first indicates that about the same fraction of the molecules present are as "active" (*i.e.*, able to transform cells to streptomycin resistance) after the second round of photoreactivation as after the first.

By contrast, at low DNA concentrations there is no competition for uptake and the number of transformants is simply proportional to the concentration of active DNA molecules. The addition of inactive DNA does not change the number of active molecules in the mixture significantly, and the transformation level in Fig. 10, therefore, remains unchanged until further photoreactivation has occurred.

From this second experiment we may decide whether or not the second round of photoreactivation occurred at about the same rate as the first. If, during every time interval in the second round of photoreactivation, the

same number of molecules per milliliter were restored to activity as during the corresponding time interval of the first round, we should expect the two halves of this curve to be identical.

As is seen, identical curves fitted to both rounds of photoreactivation in Fig. 10 fit the data within the accuracy of the experiment. The short, light, auxiliary curves represent a recovery rate just half that corresponding to the experimental curves. Clearly, if the second round of reactivation differs at all

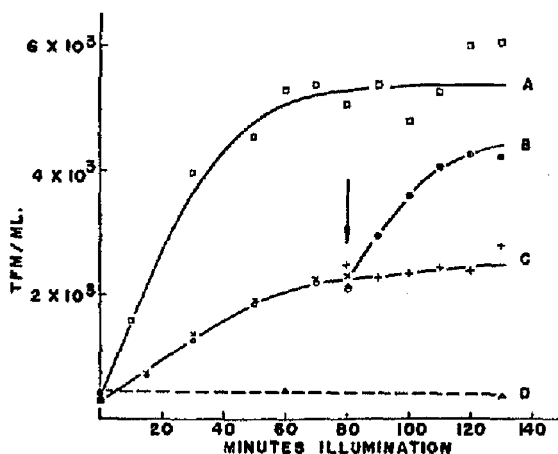

FIGURE 11. Limitation of *PR* maximum by a dialyzable component of *E. coli* extract. Mixture of 3.7 volumes UV SrDNA (1.5 γ/ml.) with 1 volume dialyzed *E. coli* extract was divided into 3 aliquots of 1.3 ml. each. Dialysate was prepared by dialyzing 2 ml. *E. coli* extract *vs.* 10 ml. 0.005 M phosphate, pH 7, overnight, lyophilizing the buffer, and redissolving in 0.5 ml. H_2O. Curve A, aliquot 1 with 0.2 ml. dialysate added at $t = 0$ minutes. Curve B, aliquot 2 with 0.2 ml. dialysate added at $t = 80$ minutes. (Open circles are for data taken before addition, and solid circles are for data taken after.) Curve C, aliquot 3 with 0.2 ml. 0.1 M phosphate, pH 7, added at $t = 80$ minutes. (Diagonal crosses are for data taken before addition and vertical crosses are for data taken after.) Curve D, control mixture of 3.5 volumes 1.5 γ/ml. *UV SrDNA* with 0.5 volume dialysate (no dialyzed extract being present).

Samples were all diluted to 1.1×10^{-3} γ UV SrDNA /ml. for assay. All UV SrDNA for this experiment was dissolved in 0.15 M NaCl, 0.014 M Na_3 citrate, 0.02 M $MgSO_4$, 0.067 M K phosphate, pH 7. The pH of the reaction mixtures was not changed by the addition of dialysate or phosphate buffer as judged by the color produced when samples (diluted tenfold in H_2O) were mixed with brom-thymol blue on a glazed porcelain plate.

from the first, it has much more than half the rate of recovery. In the light of the previous section this means that if the yeast photoreactivating agent is used up at all during the reaction, much more than half of it remains after the plateau level has been reached in this experiment.

It may be noted that the second round of photoreactivation begins with a lag like the first, regardless of the DNA concentration employed for assay.

The apparent lack of any component consumed in the yeast system contrasts with the finding previously reported for *E. coli* (2) which is supported by the additional evidence of Fig. 11. Here a mixture of UV SrDNA and lightly dialyzed *E. coli* extract has been divided into three aliquots. To one (A) concentrated dialysate is added before the start of illumination; to the second (B) it is added after 80 minutes of illumination; and to the third (C) 0.1 M phosphate at pH 7, matching the phosphate content of the dialysate, is added at 80 minutes. Evidently, in the *E. coli* case, the plateau level of recovery may represent exhaustion of a dialyzable component in the reactivating system, rather than maximal recovery of the DNA.

TABLE III

DIALYZABILITY OF YEAST PR AGENT

Crude yeast extract was diluted fivefold in 0.01 M phosphate plus 0.1 M NaCl, pH 7.1. Two ml. of this diluted extract was placed in 8/32 in. diameter cellophane dialysis tubing ("Nojax", Visking Corporation, Chicago). The bag was dialyzed for 65 hours *vs.* 1000 ml. of the diluting buffer at 5°C. in a stoppered 2 liter flask on an oscillating platform shaker. At the conclusion of the dialysis period 2 volumes of UV SrDNA (in 0.15 M NaCl + 0.066 M phosphate, pH 6.7) were mixed with 1 volume of the dialyzed diluted extract to test for photoreactivation.

A control preparation of diluted extract was held in a glass, screw-capped vial at the same temperature for the same period of time, then assayed in the same manner.

Extract and treatment	TFM per ml. from sample taken after t Min. illumination at 37°C.			
	$t = 0$	$t = 10$	$t = 20$	$t = 50$
Dialyzed fivefold diluted yeast extract	5.2×10^2	1.4×10^3	4.6×10^3	1.0×10^4
Undialyzed fivefold diluted yeast extract	5.3×10^2	1.7×10^3	5.8×10^3	1.1×10^4

Dialyzability

The PR agent of yeast is either non-dialyzable or only very slowly dialyzable as shown in Table III. Crude yeast extract diluted fivefold in a 0.01 M phosphate–0.1 M saline buffer at pH 7 and subsequently dialyzed against 500 volumes of this buffer for 65 hours with shaking showed the same activity as an aliquot of the diluted extract held undialyzed at the same temperature for the same period of time. Similarly, no activity was lost by 44 hours' dialysis of undiluted yeast extract against Tris-saline at pH 8, or 17 hours against phosphate-saline at pH 6.4. Some loss of activity occurred after 44 hours' dialysis *vs.* phosphate-saline at pH 6.1, but this could not be restored by the addition of concentrated dialysate.

An apparent decrease in yeast extract activity may be observed after dialysis outside the pH 6–7 range, providing the reaction mixture is inadequately buffered so that it reflects the dialysis pH. Apparent recovery may

then be produced by a concentrated dialysate which buffers the PR reaction mixture back toward the 6–7 region. This effect disappears when the reaction *p*H is properly controlled.

Magnitude of the Final Plateau Level

Fig. 12 shows the per cent activity of unreactivated and of maximally reactivated UV SrDNA for various doses of 254 mμ ultraviolet radiation. These two curves are related by a constant dose-reduction factor (1, 15) of about 1/10 (*i.e.*, the activity after any given ultraviolet dose with no PR is the

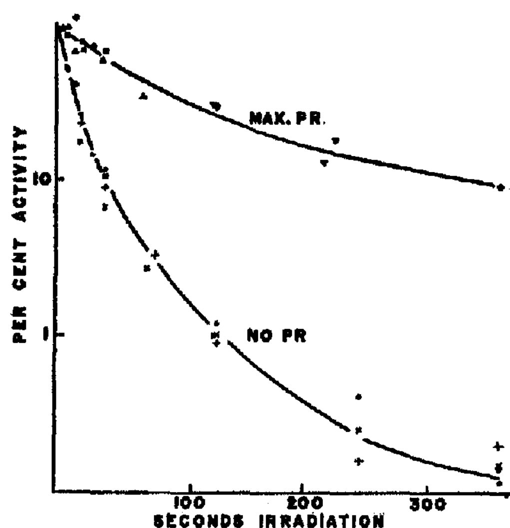

FIGURE 12. Per cent activity of UV SrDNA without photoreactivation and after maximum photoreactivation as a function of ultraviolet irradiation time. Dose rate, 25 ergs/mm.^{2}/sec.

same as the activity after about ten times that dose with maximum PR), in agreement with a finding by Goodgal (9).

Some preparations of extract have consistently given lower maxima than shown here (*e.g.*, an increase from 1 to 20 per cent instead of 30 per cent activity) regardless of the concentration at which they were employed. In such cases the recovery curve, as plotted in Fig. 7, flattened out more abruptly at its upper end than in the case of higher maximum extracts. All the data for Fig. 12 were obtained with the extracts which showed the more typical behavior.

The available data for other genetic markers of *H. influenzae* are insufficient for the construction of such curves, but it is clear that they behave qualitatively in the same manner. The plateau maximum in each case decreases as

the ultraviolet dose increases, but does so more slowly than the starting level. The maximum, therefore, becomes an increasingly larger multiple of the starting value over the dose range so far tested.

The Initial Lag

With UV SrDNA irradiated to 1 per cent of its original transforming activity, the recovery curve begins with a small positive slope which increases with illumination time to a somewhat larger value. As indicated in Fig. 5, this initial lag in recovery rate extends over longer periods of time for lower concentrations of extract, lasting until a certain level of recovery has been

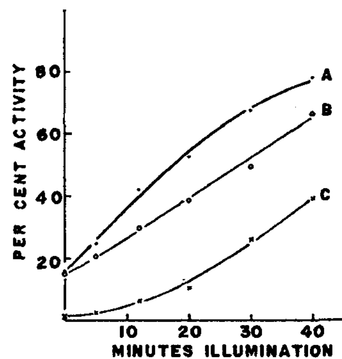

FIGURE 13. Lack of an initial lag in photorecovery for lightly inactivated UV SrDNA. Curve A, DNA of 15 per cent initial activity using 18-fold diluted yeast extract. Curve B, same DNA using 36-fold diluted extract. Curve C, DNA of 1.5 per cent initial activity using ninefold diluted extract (TFM by layer method).

attained (around 6 to 8 per cent for a 1 per cent starting level). The lag becomes less pronounced at lower radiation doses to the DNA and becomes unnoticeable when the starting activity is 10 per cent or more. These facts are illustrated by Fig. 13 showing recovery curves for 1.5 per cent and for 15 per cent active UV SrDNA.

Lower concentrations of extract are necessary with more lightly irradiated DNA or the recovery rate will be so rapid as to conceal any possible lag. Two such lower concentrations are shown here for the 15 per cent activity.

An initial lag analogous to that observed with the streptomycin resistance marker may be demonstrated for other markers, provided they have been sufficiently inactivated before photoreactivation.

Interaction of Yeast and E. coli Systems

Photoreactivation to maximum with *E. coli* extract followed by addition of yeast extract, or *vice versa*, gives a recovery curve like Fig. 8, with no further rise in activity. This observation implies that SrDNA inactivated to the 1 per cent level with ultraviolet radiation cannot be restored to more than

30 per cent activity by *E. coli* extract, since this is the maximum recovery provided in this material by yeast extract. In Fig. 2 of reference (2), a single case of restoration from 1 per cent to 50 per cent by *E. coli* extract was reported. Since no other case of so high a recovery has been observed, and since this same extract preparation when tested 6 weeks later gave a 30 per cent recovery level (as shown in the same figure), it is likely that the true recovery

TABLE IV

YEAST DIALYSATE STIMULATION OF PR IN DIALYZED E. COLI EXTRACT

Dialyzed *E. coli* extract was prepared by dialyzing *vs.* 1000 volumes of 0.1 M phosphate, 0.1 M NaCl, pH 6.8, for 16.5 hours with agitation.

Yeast dialysate (I) was prepared by dialyzing crude yeast extract *vs.* 5 volumes 0.01 M phosphate, pH 6.8, overnight, lyophilizing the dialysate, and redissolving in 0.6 volume H_2O.

Dialysate of predialyzed yeast extract (II) was prepared by predialyzing crude yeast extract *vs.* 1000 ml. 0.01 M phosphate, 0.1 M NaCl, pH 6.8, for 6.5 hours with agitation, and using the resulting material to prepare dialysate as above.

Reaction mixtures consisted of 1 volume dialyzed *E. coli* extract, 5 volumes 1.5 γ/ml. UV SrDNA (in 0.15 M NaCl, 0.013 M Na_3 citrate, 0.025 M $MgSO_4$, 0.064 M phosphate, pH 7.0), and 0.5 volume dialysate (or 0.1 M phosphate buffer, pH 6.8). The pH of reaction mixtures was 6.6–6.7, as determined by diluting samples tenfold in H_2O, mixing with brom-thymol blue on a porcelain plate, and comparing the color with similarly treated buffers.

Cell extract components in reaction mixture	Transformations per ml. produced by samples of reaction mixture after 5 min. illumination			
	$t = 0$	$t = 45$	$t = 60$	$t = 90$
Dialyzed *E. coli* extract + 0.1 M phosphate buffer	0.93×10^2	5.1×10^2	5.6×10^2	—
Dialyzed *E. coli* extract + yeast dialysate (I)	0.94×10^2	1.1×10^3	1.0×10^3	—
Dialyzed *E. coli* extract + dialysate of predialyzed yeast extract (II)	0.78×10^2	4.4×10^2	5.3×10^2	—
Yeast dialysate I (control)	1.1×10^2	—	—	1.0×10^2
Yeast dialysate II (control)	0.97×10^2	—	—	0.90×10^2

maximum from 1 per cent activity is close to 30 per cent when using *E. coli* extract.

With *E. coli* extract in which the plateau level has been lowered by dialysis, addition of dialyzed yeast extract does result in further recovery.

Concentrated dialysate of yeast extract will supplement dialyzed *E. coli* extract, raising the plateau level of recovery as shown in Table IV. The active dialyzable entity is present in roughly the same concentration in yeast extract as in *E. coli* extract, as shown by the comparative effects of diluting dialysates. This component does not appear to be closely associated with the yeast PR system, however. It is depleted by a 6.5 hour preliminary dialysis of the extract, so that concentrated dialysate subsequently prepared

from such extract does *not* supplement dialyzed *E. coli* extract. As was noted above, dialysis for ten times this long does not affect photoreactivation by the yeast extract itself.

Effect of Proteolytic Enzymes

The effect of proteolytic enzymes on the photoreactivating power of crude yeast extract is shown in Figs. 14 and 15. Incubation with solutions of either

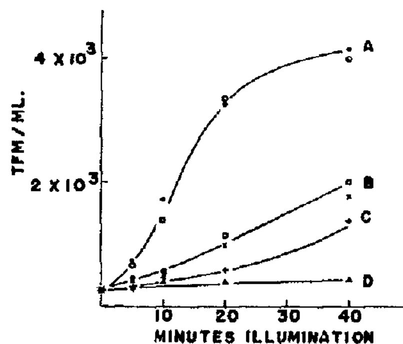

FIGURE 14. Inactivation of PR activity in yeast extract by crystalline trypsin (Worthington Biochemical Corporation, Freehold, New Jersey, twice crystallized, TR20). 0.2 ml. of yeast extract (16 mg. protein per ml.) and 0.2 ml. of trypsin solution of appropriate concentration were incubated at 37°C. for the indicated time. 0.4 ml. of 25 γ/ml. crystalline soy bean trypsin inhibitor (Nutritional Biochemical Corporation, Cleveland) was added and incubation continued to a total of 30 minutes in each case. The resulting solution was tested for PR activity as described under "Methods." Curve A, controls. Yeast extract incubated with buffer 30 minutes and diluted with buffer after incubation (solid points), and yeast extract incubated with buffer 25 minutes, soy bean inhibitor added, and incubation continued 5 minutes more (open circles). Curve B, 10 γ/ml. trypsin in digestion mixture acting for 10 minutes (squares) and 20 γ/ml. trypsin acting for 5 minutes (crosses). Curve C, 20 γ/ml. trypsin acting for 10 minutes. Curve D, 20 γ/ml. trypsin acting for 30 minutes.

crystalline trypsin or chymotrypsin destroys this power progressively at a rate which is proportional to the proteolytic enzyme concentration. As seen in Fig. 14, curve B, digestion with 20 γ/ml. trypsin for 5 minutes gives the same degree of inactivation as 10 γ/ml. for 10 minutes, and similarly (in Fig. 15, curve B), 1 γ/ml. of chymotrypsin acting for 1 hour produces the same effect as 0.5 γ/ml. acting for 2 hours. Using a fixed concentration of either proteolytic enzyme, digestion for a longer period of time gives a smaller photoreactivation rate, r (as defined in Fig. 7), implying that the concentration of the active PR agent has been correspondingly reduced. These facts suggest that the inactivation is a catalytic process rather than some

stoichiometric combination of the trypsin or chymotrypsin with components of the photoreactivation system.

In the case of trypsin, the proteolytic enzyme action was stopped at the desired time by adding crystalline soy bean trypsin inhibitor (12). This component has no direct effect on the yeast PR system, as shown by curve A of Fig. 14. In the case of chymotrypsin, low concentrations were allowed to act for 1 or 2 hours prior to the addition of ultraviolet-inactivated DNA and

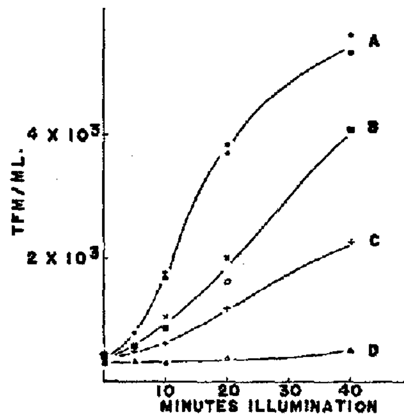

FIGURE 15. Inactivation of PR activity in yeast extract by crystalline chymotrypsin (Worthington Biochemical Corporation, Freehold, New Jersey, CD 521). 0.15 ml. yeast extract (diluted to 5.3 mg. protein/ml. in buffer) and 0.05 ml. of chymotrypsin solution were incubated for the indicated time in screw-capped test tubes, 0.4 ml. of UV SrDNA solution (1.5 γ/ml.) was added, and illumination and sampling begun. Curve A, controls. Yeast extract incubated 2 hours with buffer (solid points) and extract incubated 1 hour with 100 γ/ml. chymotrypsin which had previously been heated to 100°C. for 15 minutes (open circles). Curve B, 1 γ/ml. chymotrypsin in digestion mixture acting for 1 hour (squares) and 0.5 γ/ml. chymotrypsin acting for 2 hours (crosses). Curve C, 1 γ/ml. chymotrypsin acting for 2 hours. Curve D, 10 γ/ml. chymotrypsin acting for 1 hour (from a separate experiment).

illumination for 40 minutes to test for photoreactivation. Addition of the DNA diluted the chymotrypsin–yeast extract mixture threefold, so that digestion occurring during the 40 minute PR test was not over about ⅙ to ⅔ that occurring prior to the test.

Appropriate controls demonstrated that the trypsin and chymotrypsin solutions did not themselves inactivate transforming DNA, showing that no pancreatic DNase was present in sufficient concentration to complicate the interpretation of these experiments. Another control showed that the peptides and amino acids resulting from the digestion of yeast extract proteins probably do not inhibit the PR system to give the observed inactivation. Yeast extract inactivated by 100 γ/ml. of trypsin or chymotrypsin for 30 minutes was

heated to destroy the proteolytic activity and used to dilute fresh yeast extract by tenfold. Such diluted extract was fully active. It appears, therefore, that the yeast PR agent is itself inactivated by the enzymatic action of trypsin and chymotrypsin.

TABLE V

SALTING-OUT AND RECOVERY OF YEAST PR AGENT

A 5°C. saturated solution of ammonium sulfate was titrated to pH 6.6 with NaOH. Three ml. of crude yeast extract was brought to 45 per cent saturation in ammonium sulfate by adding 0.82 volume of this stock. The precipitate was centrifuged off and redissolved in cold H_2O. The supernatant was brought to 55 per cent saturation by adding 0.22 volume of ammonium sulfate stock, and the precipitate again centrifuged off and redissolved in cold H_2O. Each preparation listed in the table below was dialyzed *vs.* 200 volumes of 0.01 M phosphate at pH 6.6, and tested as described under Materials and Methods after dilution by the indicated amount (TFM by layer method). The photoreactivation rate, *r*, was measured as indicated by Figs. 6 and 7 and the associated text discussion.

Preparation	Volume (ml.)	Dilution before PR	PR rate (r)	Relative concentration of PR agent ($r \times$ dilution)	Relative amount of agent present	
					Concentration × volume	Per cent
Crude yeast extract	3	8	1.00*	8.0	24.0	100*
Precipitate at 45 per cent saturation redissolved	1.2	4	0.85	3.4	4.1	17
Precipitate at 55 per cent saturation redissolved	1.2	12	1.35	16.2	19.5	81
Supernatant at 55 per cent saturation	6.6	Undiluted	0.55	0.55	3.7	15
					Recovered,	113

* By definition.

Salting-Out and Recovery of Yeast PR Agent

The photoreactivating agent of yeast extract may be salted out of solution and recovered quantitatively as shown by the data of Table V.

The extract was treated successively with ammonium sulfate at 45 per cent and 55 per cent saturations, and the centrifuged precipitates were redissolved and dialyzed overnight, along with the 55 per cent supernatant and a sample of the starting extract. Appropriate dilutions of these preparations were used to photoreactivate 1 per cent active UV SrDNA, and the relative rate, r (as defined in Fig. 7), was measured for each preparation. This rate was multiplied by the dilution to give the relative concentration of PR agent in each preparation and the relative amount of the agent was computed from this concentration and the volume of each preparation. Total recovery in this experiment was 113 per cent of the starting amount. In two similar experi-

ments, using different ammonium sulfate concentrations, it was 80 per cent and 91 per cent.

DISCUSSION

Photoreactivation by yeast extract evidently results in the same type of repair as that provided by *E. coli* extract. Not only are the two kinds of reactivation qualitatively similar but DNA reactivated to the greatest degree possible by either system cannot be further reactivated by the other.

The yeast system differs from that of *E. coli* in its apparent lack of any component which is used up during photoreactivation. With *E. coli* extract, the maximal level of recovery can be limited by limiting the amount of dialyzable component present. Inactivated DNA, which has been reactivated to an apparent maximum in the presence of a limiting amount of this component, will recover further if more is added. This is most simply interpreted as meaning that the dialyzable component is irreversibly used up during illumination. With yeast extract, on the other hand, DNA reactivated to a maximum does not recover further on addition of either more yeast or more *coli* extract, and the yeast extract remaining in a reaction mixture after maximal reactivation is capable of reactivating additional ultraviolet-inactivated DNA at about the same rate and to about the same extent as the first. In this latter case, at least one component of the PR agent is not present in great excess, because decreasing the extract concentration decreases the recovery rate proportionately.

Even better evidence that the yeast PR agent is not used up is provided by the phenomenon of competitive inhibition (6), which will be considered in detail in a separate paper. DNA from a variety of sources which is incapable of producing bacterial transformations may be added to a photoreactivation mixture without effect on the photorecovery of ultraviolet-inactivated transforming DNA. If this added DNA has first been ultraviolet-irradiated, however, the rate of recovery is slowed down. The observed inhibition increases with the amount of irradiated non-transforming DNA and with the ultraviolet dose delivered to it. Inhibition may be diminished by allowing the yeast extract to act on the competing DNA in the light prior to addition of the ultraviolet-irradiated transforming DNA. With sufficient preillumination it disappears entirely, and the recovery rate is indistinguishable from the rate observed with unirradiated non-transforming DNA or without non-transforming DNA. In this phenomenon, PR activity of the extract which is unavailable in the presence of competing ultraviolet-irradiated DNA becomes available again simply by allowing the extract to act on the competing material first. This strongly suggests a catalytic action of the yeast PR agent.

Preparations of yeast agent purified over one hundred times, in terms of activity per unit protein concentration, produce reactivation in the same manner and to the same degree as crude material without any necessity for recombining fractions. It is perhaps possible that a factor analogous to the dialyzable component of the *E. coli* system exists, but that the purification procedures have failed to separate it from other components of the system. However, no experimental evidence suggesting that this is the case has been found.

The yeast agent is non-dialyzable, and heat-labile. It is progressively inactivated by solutions of crystalline trypsin or chymotrypsin at rates which increase with proteolytic enzyme concentration, and it may be salted out of the extract and recovered quantitatively in active form. These characteristics suggest a protein, and since the agent evidently catalyzes some type of photochemical repair of the ultraviolet-damaged DNA, it seems reasonable to call it an enzyme.

This photoreactivating enzyme (PRE) is interesting for two reasons. First, it is a photoenzyme, and few enzymes involved in photochemical reactions are known at present. Second, it acts on DNA *in vitro* without depolymerizing it. Aside from the enzyme system of Kornberg and his coworkers (13), which synthesizes polydeoxyribonucleotide, yeast PRE (together with its *E. coli* analogue) is the only enzyme known which has a "constructive" action on DNA outside an intact cell.

The quantitative relationship, $T = F(rt)$, which exists between the transforming activity, T, and the illumination time, t, for different enzyme concentrations (Figs. 5–7) is most readily explained by supposing that a given amount of chemical repair in the damaged molecules will result in a definite amount of recovered activity. If r is proportional to the rate of this elementary chemical reaction, the product rt should determine the total amount of repair effected to time, t. F, on the other hand, describes the way in which transforming activity increases with various total amounts of repair. According to this picture F should not depend on enzyme concentration, which affects only r, but could change with the ultraviolet dose applied to the DNA. This is in accord with the experimental facts.

We must assume two classes of ultraviolet damage—one capable of photoreactivation and the other not—in order to account for the observed recovery maximum of less than 100 per cent activity. There is no experimental basis at present for deciding whether these classes represent essentially different kinds of photochemical lesions produced by the ultraviolet radiation, or whether the difference is one of location or some similar factor making this damage susceptible or not to the enzyme action.

The initial lag in recovery could be attributed either to a delay in the start of repair by the enzyme system after the application of light or to the neces-

sity for a certain minimum amount of repair before any recovery of activity occurs. The latter effect would be expected if the sensitive portions of the molecules could sustain multiple lesions, all of which required repair in order to restore activity. In such a case even if the elementary chemical repair proceeded from the beginning of illumination, fully repaired units would not appear in appreciable numbers until after some period of time had passed. The evidence with the yeast PR system favors this latter view. After the illumination is interrupted for a period (Fig. 2), recovery resumes without delay upon reapplication of light. On the other hand, after reactivation to maximum and addition of more UV SrDNA (Figs. 9 and 10), the "second round" of photoreactivation begins with a lag like the first. The magnitude of this lag decreases with decreasing ultraviolet dose to the DNA.

The target theory of Atwood and Norman (14) provides a basis for further investigation of this possibility, bearing in mind that these authors were concerned with the random *inactivation* of cells having varying numbers of radiation-sensitive units (*e.g.*, nuclei) while we are concerned with the random photochemical *reactivation* of genetic marker units containing varying numbers of ultraviolet lesions. With appropriate changes in terminology necessitated by this difference, their analysis applies directly. According to it, if N_o is the number of active molecules before photoreactivation, N_m, the number after photoreactivation to maximum, and N the number at some intermediate state (after t minutes illumination), a plot of $[\log (N_m - N)/(N_m - N_o)]$ *vs.* t should give a curve which becomes a straight line as N approaches N_m. Extrapolating this straight line segment to $t = 0$ should give the logarithm of the mean number of repair steps necessary to restore activity to one of the marker units. No assumptions are made about the numerical distribution of lesions among the genetic marker units in this analysis.

Unfortunately, the accuracy with which it can be applied to the available data is low. Assuming that transforming activity is proportional to the number of molecules carrying the genetic marker unit in active form, the portions of the logarithmic straight line segment which have most influence on the extrapolation are those for which $(N_m - N)$ is small and for which experimental errors consequently introduce large uncertainties. The data for the streptomycin resistance marker indicate that for an initial activity of 10 per cent or more the process is essentially single step, for an initial activity of 1 per cent the multiplicity is perhaps 2–3, and for heavier ultraviolet doses is still larger. Further discussion is, however, unwarranted in the absence of more extensive data.

A lag period is observed in photoreactivation by *E. coli* extract similar to that seen with yeast when the dialyzable and non-dialyzable components have been separated and recombined (see reference (2), Figs. 8 and 9). The lag is never noticeable, however, in photoreactivation by untreated *E. coli*

extract. The kinetics of this two (or more) component system offer enough possibilities of complication so that there is no clear contradiction in this at present. We do not know (to cite one example) whether the dialyzable component is immediately available in the undialyzed *E. coli* extract, or whether it is supplied to the photoreactivation system by some auxiliary reaction. Any such preliminary step could markedly affect the initial shape of the photorecovery curve.

The picture of photoreactivation developed in the foregoing discussion is conveniently summarized by the schematic diagram of Fig. 16. Among

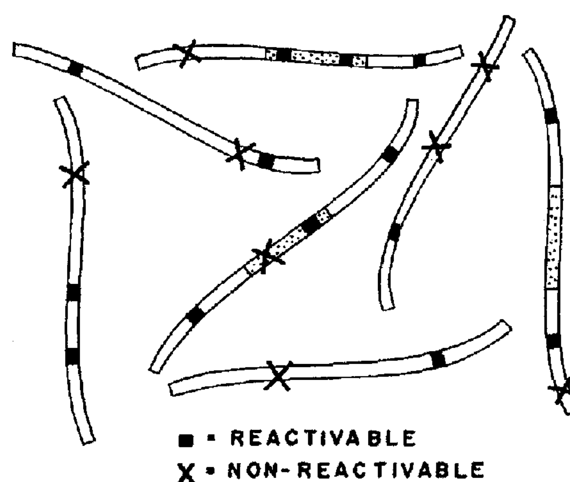

FIGURE 16. Schematic representation of a population of ultraviolet-irradiated molecules of transforming DNA.

the total population of molecules in a preparation of transforming DNA, some (marked with a stippled region) carry the particular marker being assayed. Two classes of photochemical damage are found sprinkled through this molecular population after irradiation by ultraviolet. One class is subject to reversal by photoreactivation and the other is not.

Some of the stippled regions—the radiation-sensitive areas associated with genetic locus of the marker in question—have escaped all damage. These are pictured as responsible for the residual transforming activity of the DNA after irradiation. Some of these sensitive areas have suffered irreversible damage and the corresponding locus will be incapable of conveying its genetic character into a cell even after photoreactivation. These represent the portion of the original transforming activity which is not reactivable. Finally, some have suffered only reversible damage. These represent transforming activity which can be restored. With sufficient ultraviolet dose such radiation-sensitive regions will, on the average, have suffered multiple

damage, and until all such damage is repaired they will remain inactive. Hence, the "multi-hit" character of the recovery curves. At present there is no evidence to identify the radiation-sensitive region with the genetic locus itself (*i.e.*, the region which must be incorporated into the cell's genome to convey the genetic trait in question). We should expect the radiation-sensitive region to include this locus, but damage at other points in the molecule may also diminish the probability of a successful transformation. The sensitive region could, therefore, be larger than the locus.

In terms of the picture presented here the problem of photoreactivation is to determine the chemical nature of the reactivable ultraviolet lesions and the mechanism of enzyme action by which they are repaired in restoring normal functional activity to DNA.

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